International Journal of Antimicrobial Agents 47 (2016) 77–83

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: http://www.elsevier.com/locate/ijantimicag

# Increase of efflux-mediated resistance in *Pseudomonas aeruginosa* during antibiotic treatment in patients suffering from nosocomial pneumonia☆

Mickaël Riou[a,1,4], Laëtitia Avrain[b,4], Sylviane Carbonnelle[a,2], Farid El Garch[a,c,3], Jean-Paul Pirnay[d], Daniel De Vos[d], Patrick Plésiat[e], Paul M. Tulkens[a], Françoise Van Bambeke[a,*]

[a] *Pharmacologie cellulaire et moléculaire & Louvain Drug Research Institute, Université catholique de Louvain, Brussels, Belgium*
[b] *Coris BioConcept, Gembloux, Belgium*
[c] *Laboratoire de microbiologie, CHU Dinant-Godinne UCL Namur, Yvoir, Belgium*
[d] *Laboratory for Molecular and Cellular Technology, Queen Astrid Military Hospital, Neder-over-Heembeek, Brussels, Belgium*
[e] *Laboratoire de bactériologie, Faculté de médecine, Université de Franche-Comté, Besançon, France*

ARTICLE INFO

*Article history:*
Received 13 August 2015
Accepted 4 November 2015

*Keywords:*
*Pseudomonas aeruginosa*
Resistance
Efflux
MexAB–OprM
MexXY–OprM
EUCAST breakpoints

ABSTRACT

Increases in antibiotic minimum inhibitory concentrations (MICs) for *Pseudomonas aeruginosa* during treatment are commonly observed but their relationship to efflux overexpression remains poorly documented. In this study, pairs of first [at time of diagnosis (D0)] and last [during treatment (DL)] *P. aeruginosa* isolates were obtained from patients treated for suspicion of nosocomial pneumonia. Pair clonality was determined by repetitive extragenic palindromic PCR. Overexpression of *mexA* and *mexX* was assessed by real-time PCR, and expression of *mexC* and *mexE* was assessed by PCR. Antibiotics received by patients before and during treatment were determined from clinical charts. For D0 isolates, 24% were from patients without antibiotics for 1 month and 64% were negative for *mexA*/*mexX* overexpression and *mexC*/*mexE* expression. For DL isolates, approximately one-half of the patients had received piperacillin/tazobactam, amikacin, meropenem and/or cefepime, and 17% had received ciprofloxacin (alone or in combination); 38% did not show changes in expression of the four genes, whereas 38% showed increased expression for one gene (mainly *mexA* or *mexX*), 19% for two genes (mainly *mexA* and *mexX*) and 5% for three or four genes. Isolates overexpressing *mexA* or *mexX* had median MICs above EUCAST clinical resistance breakpoints for ciprofloxacin, cefepime and meropenem, or for ciprofloxacin, amikacin, cefepime and meropenem, respectively. *mexA* or *mexX* overexpression was statistically significantly associated with patients' exposure to ciprofloxacin and meropenem or cefepime and meropenem, respectively. Overexpression of genes encoding antibiotic transporters in *P. aeruginosa* during treatment is frequent and is associated with increases in MICs above EUCAST clinical susceptibility breakpoints.


## 1. Introduction

With its large genome, *Pseudomonas aeruginosa* shows a remarkable adaptability for surviving under antibiotic selection pressure [1]. Although the emergence of resistance in *P. aeruginosa* during antibiotic treatment has been described since the mid 1980s [2], documentation of its mechanisms remains scarce. In this context, we collected successive clonally related isolates of *P. aeruginosa* from patients hospitalised in intensive care units (ICUs) with clinically suspected nosocomial pneumonia and receiving one or several commonly used antipseudomonal antibiotics [piperacillin/tazobactam (TZP), cefepime, meropenem, amikacin and ciprofloxacin]. We previously reported a markedly decreased susceptibility occurring during therapy for each of these antibiotics

☆ Portions of this work were presented at the 20th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Vienna, Austria, 10–13 April 2010 (abstract no. P780).

* Corresponding author. Present address: Pharmacologie cellulaire et moléculaire, Université catholique de Louvain, Avenue E. Mounier 73 B1.73.05, B-1200 Brussels, Belgium. Tel.: +32 2 764 7378.
*E-mail address:* francoise.vanbambeke@uclouvain.be (F. Van Bambeke).
[1] Present address: Institut National de Recherche Agronomique (INRA), Centre Val de Loire, site de Tours, UE-1277 Plateforme d'Infectiologie Expérimentale (PFIE), Nouzilly, France.
[2] Present address: Federal Agency for Nuclear Control, Brussels, Belgium.
[3] Present address: Vétoquinol SA, Lure, France.
[4] These two authors contributed equally to this study.

http://dx.doi.org/10.1016/j.ijantimicag.2015.11.004
0924-8579/

that was correlated in a statistically significant manner with the proportion of their use when examined at the whole population level [3].

The present report builds on the observation that efflux-mediated resistance can be upregulated in vitro upon incubation of bacteria with substrates of the corresponding transporters (see, e.g., [4]). Efflux can decrease the activity of all commonly used antipseudomonal drugs, namely β-lactams (substrates of MexAB–OprM; cefepime also being a substrate of MexCD–OprJ and MexXY–OprM, and piperacillin and meropenem are also marginally transported by MexCD–OprJ), aminoglycosides (substrates of MexXY–OprM) and ciprofloxacin (substrate of all three abovementioned transporters and of MexEF–OprN) (see [5] for a review). Up to now, overexpression of antibiotic efflux transporters in *P. aeruginosa* (mainly MexXY–OprM) during treatment has been only clearly demonstrated in clinical isolates collected from cystic fibrosis patients receiving repeated courses of antibiotics (see, e.g., [6] and references cited therein). Using clonally related pairs of *P. aeruginosa* isolates, we show here that overexpression of efflux is frequent and is associated with increases in minimum inhibitory concentrations (MICs) to levels above the current European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) clinical susceptibility breakpoints for the antibiotics that are substrates of the corresponding transporters.

## 2. Materials and methods

### 2.1. Patient selection and bacterial isolates

The study protocol was approved by the Ethical Committee of the Faculty of Medicine of the Université catholique de Louvain (Brussels, Belgium) within the context of a grant application to the Belgian Fonds de la Recherche Scientifique Médicale (F.R.S.M.). The patient selection criteria have been described in detail previously [3]. In brief, samples were obtained from 59 patients hospitalised in the ICUs of five teaching hospitals in Belgium with a diagnosis (or suspicion) of nosocomial pneumonia and for whom *P. aeruginosa* could be isolated from endotracheal aspirate, bronchoalveolar lavage, pleural fluid, empyema or blood and was considered as the most likely cause of the disease (see [3] for detailed diagnostic criteria). A first sample (D0 sample) was obtained at the time of initial diagnosis and additional samples were collected during the course of therapy. In the present study, we only considered this first sample and the last usable sample of each patient (DL sample) (no. of days after collection of D0 sample: range, 1–123 days; mean, 23 days; median, 17.5 days) [3]. The methods used for identification of the isolates and for assessment of clonal relatedness of strains within paired isolates (D0 vs. DL samples) have been described previously [3]. This allowed for the identification of 62 clonal pairs of strains in the 59 patients. For three patients, we observed the presence of two different strains both in D0 and DL samples that were clonally distinct from each other in both samples but were clonally related when comparing the same strain between the D0 and the DL samples [3]. *Pseudomonas aeruginosa* ATCC 27853 (American Tissue Culture Collection, Manassas, VA) and PAO1 [7] reference strains were used as quality control for susceptibility testing and as reference for standardisation of gene expression levels, respectively. All strains were kept at −80 °C in 20% glycerol–80% cation-adjusted Muller–Hinton broth (CA-MHB) until analysis and were grown overnight at 37 °C on tryptic soy agar plates (Becton Dickson, Franklin Lakes, NJ) before being used for experiments.

### 2.2. Antibiotic prescription data

Data on antibiotics prescribed to the patients at the time of pneumonia diagnosis and up to 1 month before the onset of pneumonia were collected from clinical charts. The doses, schedules and mode of administration were checked against the corresponding standards of care and were, for most of the patients, those recommended for severe infections based on the applicable Summary of Product Characteristics [3].

### 2.3. Minimum inhibitory concentration determination and susceptibility interpretive criteria

MICs were determined by geometric microdilution in CA-MHB according to CLSI recommendations [8]. Susceptibility was assessed according to both the 2014 CLSI [8] and 2015 EUCAST (http://www.eucast.org/) interpretive criteria.

### 2.4. Preparation of cDNA

#### 2.4.1. Culture of strains

Two to three colonies of each overnight culture were suspended in 10 mL of sterile MHB, were diluted to obtain an optical density at 620 nm ($OD_{620}$) of 0.1, and were incubated at 37 °C under aerobic conditions and shaking at 150 rpm until reaching an $OD_{620}$ of $1.0 \pm 0.1$. Two aliquots of 1.5 mL were taken and were centrifuged at $5000 \times g$ for 5 min at 4 °C. After discarding the supernatants, pellets were stored at −80 °C until analysis.

#### 2.4.2. Extraction of total RNA

After sample thawing, RNA was extracted using an RNeasy® Kit (QIAGEN, Hilden, Germany) following the manufacturer's recommendations. Bacterial pellets (ca. $5 \times 10^8$ CFU/100 μL) were re-suspended in 100 μL of TE buffer [10 mM Tris–HCl, 1 mM ethylene diamine tetra-acetic acid (EDTA), pH 8] containing 0.4 mg/mL lysozyme (Sigma–Aldrich, Saint Louis, MO) and were incubated at room temperature for 10 min. Lysis was achieved by adding 350 μL of commercial lysis Buffer RLT (QIAGEN) supplemented with 1% β-mercaptoethanol (Bio-Rad, Hercules, CA) and vigorous shaking, after which 250 μL of ethanol (96–100%) was added to the lysate, followed by shaking through pipetting. The suspension (usually 700 μL) was put on an RNeasy® Mini Spin Column (QIAGEN) and was treated exactly as recommended by the manufacturer. The extracted RNA was stored at −80 °C before use.

#### 2.4.3. RNA purification, quantification and retrotranscription

After thawing, extracted RNA was treated with Turbo DNase I (Ambion, Austin, TX) to eliminate possible contamination with DNA. A mix containing 2 μL of enzyme, 10 μL of RNA, 5 μL of ad hoc buffer and 33 μL of water was incubated for 30 min at 37 °C, after which an additional 2 μL of enzyme was added to the samples, which were then incubated for another 30 min at 37 °C. The reaction was stopped by incubation with 10 μL of DNAse Inactivation Reagent (Ambion) for 2 min, after which samples were centrifuged at 14 000 rpm for 2 min and supernatants were collected and stored at −80 °C. Absence of contaminating DNA was checked by performing a control PCR amplification of the *rpsL* housekeeping gene using the primers rpsL-F (CGGCACTGCGTAAGGTATGC) and rpsL-R (CGTACTTCGAACGACCCTGCT). The PCR programme consisted of 10 min at 95 °C, 40 cycles of 1 min at 95 °C, 1 min at 60 °C and 1 min at 72 °C, followed by 10 min 72 °C. The absence of an amplicon after gel electrophoresis was checked for. RNA was quantified using a Qubit® RNA BR Assay Kit (Invitrogen, Carlsbad, CA) as described by the manufacturer. RNA was then amplified using a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Penzberg, Germany) following the manufacturer's instructions, except that only 500 ng of RNA was used as starting material instead of 1 μg for optimisation reasons.

### 2.5. PCR amplification of mexC and mexE

PCR amplification was performed on 5 μL of a 1:10 dilution of purified cDNA in a final volume of 25 μL using published primers for *mexC* (mexC3 and mexC4) and *mexE* (mexE4 and mexE5) [9] and those described above for *rpsL* (used as a positive control for each PCR reaction) and the following amplification programme: 20 s at 95 °C, 20 s at 60 °C (*mexC* and *rpsL*) or 61 °C (*mexE* and *rpsL*), and 30 s at 72 °C. Amplification products were analysed by agarose gel electrophoresis for detection of the expected PCR fragments.

### 2.6. Real-time PCR amplification of mexA and mexX

The expression levels of *mexA* and *mexX* were quantified using a real-time PCR *mex* Q-TesT® (Coris BioConcept, Gembloux, Belgium) and iQ™ SYBR® Green Supermix (Bio-Rad) following the manufacturer's instructions and starting from ca. 5 ng of cDNA in a final volume of 25 μL. Relative expression levels were calculated according to Pfaffl's equation [10] where PCR efficiency was calculated based on a standard curve (included in the *mex* Q-TesT® kit) performed at the same time for each gene, after normalisation by two undisclosed housekeeping genes included in the kit (to validate the RNA isolation and the efficiency of the reverse transcription of the RNA into cDNA) and by reference to the expression level measured for the reference strain PAO1. According to instructions of the kit's manufacturer, overexpression was considered statistically significant when reaching, respectively, 2-fold (*mexA*) and 5-fold (*mexX*) the level measured for PAO1. All assays were made in triplicate on two to three independent cDNA preparations.

### 2.7. Statistical analyses

Statistical analyses were performed using GraphPad InStat v.3.06 and GraphPad Prism v.4.3 and v.6.05 (GraphPad Software, La Jolla, CA).

## 3. Results

### 3.1. Antibiotics received by the patients

Table 1 summarises the antibiotics administered to the patients and included in the analysis (see also [3] for details about combination therapies and the suitability of antibiotic dosages and schedules for this type of infection; four patients also received other antibiotics [ceftazidime, aztreonam, ceftriaxone, gentamicin or colistin] that were not included in the present analysis). For the D0 samples, (i) approximately one-third were from patients who had not received any antibiotics during the preceding 1 month, (ii) another one-third were from patients having received TZP or amikacin and (iii) approximately one-quarter were from patients having received meropenem or cefepime (only a few patients had received ciprofloxacin). For the DL samples, most patients had been exposed to at least one of the antibiotics included in the analysis. When considering exposure over the whole time period of the study (thus either before or after the diagnosis of pneumonia), the majority of the isolates were derived from patients having received at least one β-lactam, one-half from patients having received amikacin and approximately one-fifth from patients having received ciprofloxacin. Most patients had been treated with more than one antibiotic, either in succession or in combination.

### 3.2. Changes in efflux transporter expression during treatment

Fig. 1 shows the proportion of initial (D0) and last (DL) isolates overexpressing *mexA* or *mexX* in comparison with the basal expression level in wild-type strains, or expressing *mexC* or *mexE* (no expression in wild-type strains), or a combination thereof. Considering first the 62 D0 isolates, 40 (65%) did not show increased gene expression of efflux transporters compared with PAO1 (no overexpression of *mexA* or *mexX*; no detectable expression of *mexC* or *mexE*), whilst 15 (24%) showed increased expression of a single gene (4 for *mexA*, 6 for *mexX*, 4 for *mexC* and 1 for *mexE*) and 7 (11%) showed increased expression of two genes (simultaneous overexpression of *mexA* and *mexX* in 6 cases); no isolates showed overexpression of three or four genes. Regarding the 62 DL isolates, only 24 (39%) were without increased gene expression of efflux transporters compared with PAO1, 23 (37%) showed

**Table 1**
Number of patients having received at least one dose of one or several of the antibiotics examined in this study (i) before collection of the initial isolate (D0) or (ii) during the period between collection of the initial isolate and collection of the last isolate (DL). Figures in bold and on a grey background show the total number of patients having received the corresponding antibiotic. Plain figures on a white background above (column) or on the right (row) of this bold number show the number of patients having also received another antibiotic [as indicated in the corresponding extreme left column (when moving along the column) or the corresponding top row (when moving along the row)]. None means no additional antibiotic (monotherapy or no antibiotic treatment).[a]

| Antibiotic | CIP | MEM | TZP | AMK | FEP | None |
|---|---|---|---|---|---|---|
| Initial isolate (D0) | | | | | | |
| CIP | **5** | 3 | 2 | 4 | 2 | 1 |
| MEM | | **15** | 7 | 10 | 6 | 2 |
| TZP | | | **18** | 11 | 5 | 6 |
| AMK | | | | **19** | 4 | 4 |
| FEP | | | | | **14** | 6 |
| None | | | | | | **24** |
| Last isolates (DL) | | | | | | |
| CIP | **11** | 5 | 5 | 9 | 5 | 0 |
| MEM | | **28** | 17 | 13 | 12 | 3 |
| TZP | | | **31** | 17 | 14 | 4 |
| AMK | | | | **29** | 17 | 4 |
| FEP | | | | | **29** | 7 |
| None | | | | | | **5** |

CIP, ciprofloxacin; MEM, meropenem; TZP, piperacillin/tazobactam; AMK, amikacin; FEP, cefepime.
The sum of the plain numbers above or on the right of each bold number may not correspond to this bold number because some patients received more than two antibiotics

[a] As an example, for D0 isolates, 18 patients received TZP (see figure in bold), amongst whom 7 also received MEM and 2 CIP (see plain figures in the column above), while 11 also received AMK, 5 FEP and 6 no other antibiotic (monotherapy) (see plain figures in the row to the right).

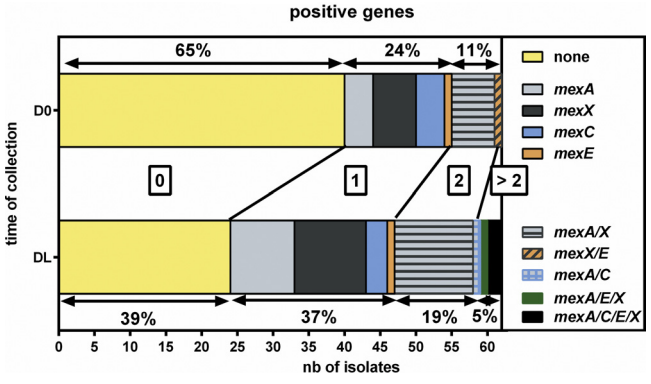


**Fig. 1.** Number of isolates expressing genes encoding for efflux systems according to their time of collection (D0, initial isolates; DL, last isolates). The following criteria were used: for *mexA* and *mexX* (constitutively expressed), a 2- and 5-fold increase in overexpression of levels observed in real-time PCR amplification compared with the reference strain PAO1; for *mexC* and *mexE*, detection of a positive signal in agarose gel electrophoresis at the position of the expected PCR fragments. Square figures indicate the number of efflux systems detected, with the corresponding percentages of isolates indicated above (D0) or below (DL) the bars.

increased expression of a single gene (overexpression of *mexA* in 9 cases and *mexX* in 10 cases), 12 (19%) showed increased expression of two genes (*mexA*/*mexX* in 11 cases), 1 (2%) showed increased expression of three genes (*mexA*/*mexX*/*mexE*) and 2 (3%) showed increased expression of all four genes.

### 3.3. Relationship between efflux pump expression and susceptibility to substrate antibiotics

Fig. 2 shows box-plot analyses of the MIC distributions of the five antipseudomonal antibiotics examined for all isolates when

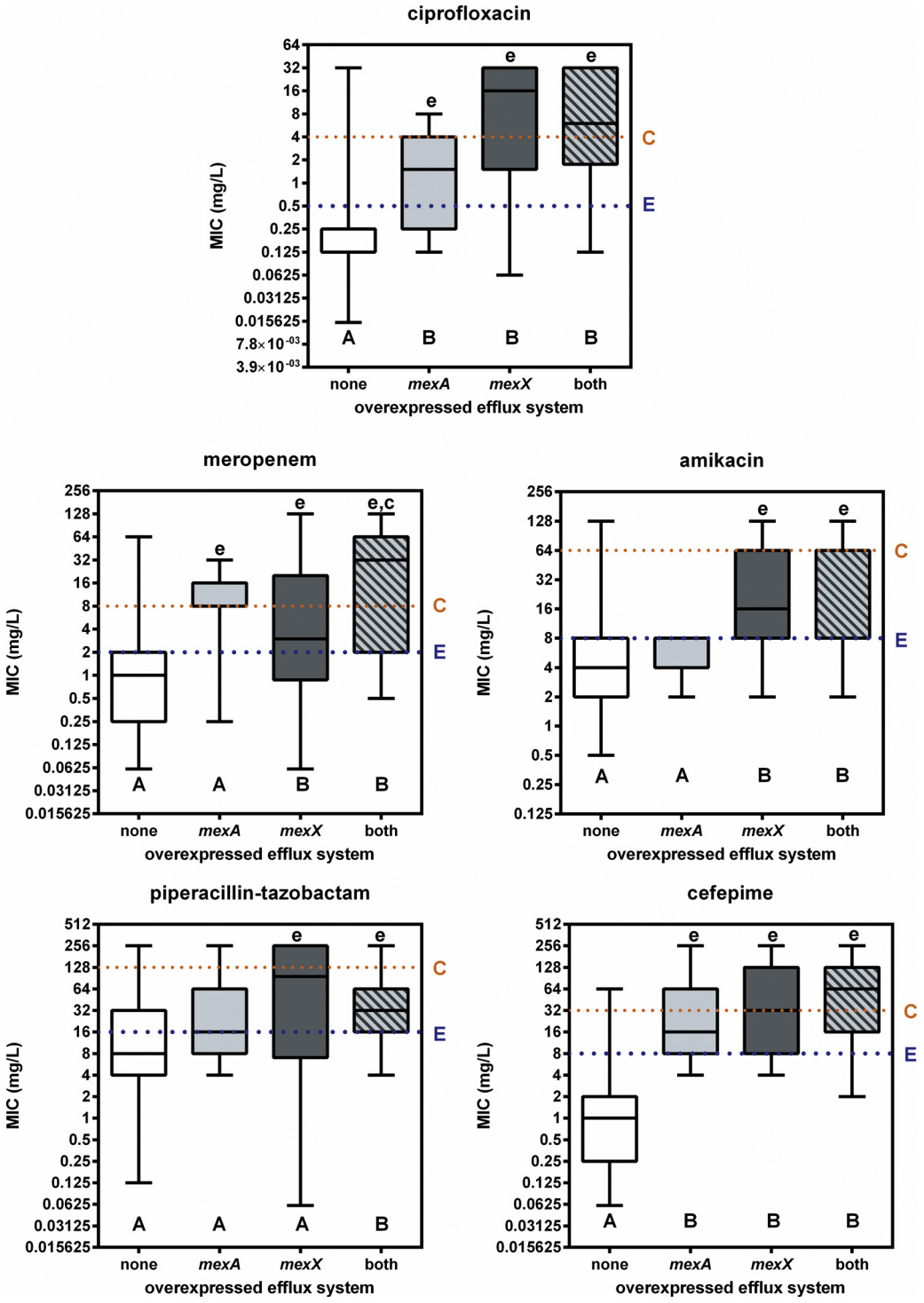


**Fig. 2.** Box and whiskers plots of the minimum inhibitory concentrations (MICs) of the five antipseudomonal antibiotics examined for all isolates (initial and last) according to the expression status of *mexA* and *mexX* genes. The two horizontal dotted lines indicate the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (blue line [marked E]) and Clinical and Laboratory Standards Institute (CLSI) (orange line [marked C]) clinical susceptibility breakpoints, respectively (clinical resistance being defined as a microbial with an MIC value of the recommended antibiotic higher than the corresponding EUCAST value and higher than or equal to the corresponding CLSI value). Statistical analyses: (i) whole distribution analysis according to the efflux transporter overexpressed: blocks with different upper case letters (located at the bottom of each block) correspond to statistically different MIC distributions (one-way ANOVA; Kruskal–Wallis test; Dunn's multiple comparison test); (ii) comparison of median MICs with clinical breakpoints: lower case letters (located at the top of the block) indicate which median MIC value is statistically significantly larger than the EUCAST (e) or CLSI (c) clinical breakpoint value (*t*-test).

stratified based on the overexpression of *mexA* and *mexX*, for which a large proportion of the collection was positive. Overexpression of *mexA* was accompanied by a statistically significant increase in the overall MIC distributions of ciprofloxacin and cefepime, with median MICs being statistically significantly higher than the EUCAST clinical breakpoints for these two antibiotics. For meropenem, the median MIC also exceeded the EUCAST clinical breakpoint but the MIC distribution was not statistically different from that of strains with basal expression of *mexA*, essentially due to the large spread of the individual values in both cases. Overexpression of *mexA* was also associated with a shift in the MIC distribution and in the median MIC of TZP, but this was not statistically significant. Overexpression of *mexX* was associated with a statistically significant increase in the overall MIC distributions of ciprofloxacin, amikacin, cefepime and meropenem (but again with a large spread of individual values for the latter antibiotic), and median MICs were statistically significantly higher than the EUCAST clinical breakpoints for all four antibiotics. Although there was no statistically significant increase in the MIC distribution for TZP for isolates overexpressing *mexX*, the median MICs of these strains reached a value that was statistically significantly larger than the EUCAST clinical breakpoint. Isolates for which both *mexA* and *mexX* were overexpressed showed a reduced susceptibility to all five tested antibiotics when considering MIC distributions (with no apparent difference from isolates overexpressing *mexX* only for ciprofloxacin, amikacin, meropenem and cefepime, and no apparent difference from isolates overexpressing *mexA* only for cefepime), with median MICs exceeding the EUCAST clinical breakpoints for all five antibiotics and the CLSI breakpoint for meropenem.

### 3.4. Relationship between antibiotic exposure and overexpression of efflux systems

Fig. 3 shows the risk factors for patients to harbour a *P. aeruginosa* strain overexpressing *mexA* or *mexX* at the last day of sampling (DL) when having been administered one of the five antipseudomonal antibiotics examined in this study. Overexpression of *mexA* was associated in a statistically significant manner with exposure of the patient to ciprofloxacin or meropenem. Overexpression of *mexX* was more frequent but was not statistically significant even in samples obtained from patients having received ciprofloxacin or amikacin. The time at which the antibiotics were administered (before or after diagnosis of pneumonia) did not affect the results.

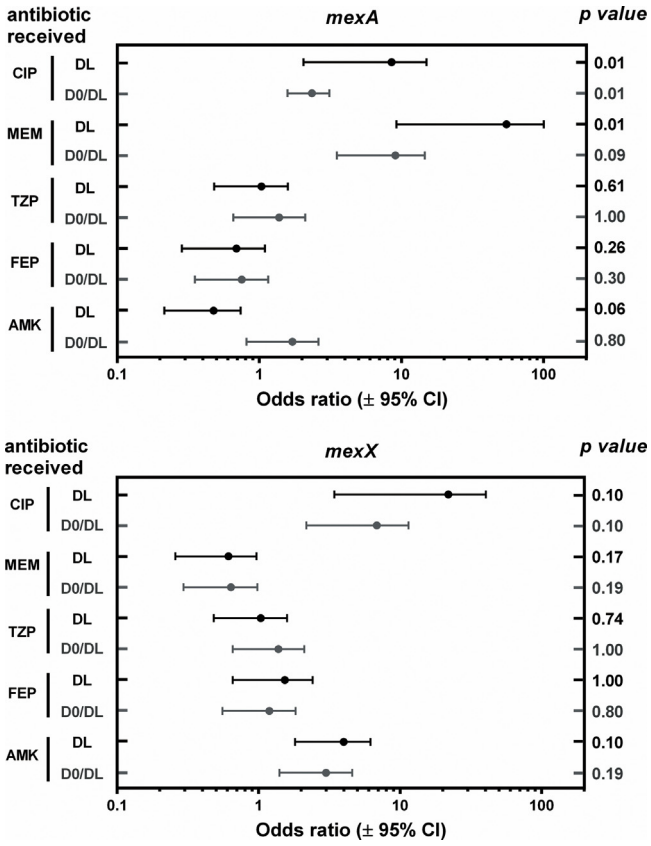


**Fig. 3.** Estimated effect [odds ratio ± 95% confidence interval (CI)] of previous antibiotic exposure on overexpression of *mexA* or *mexX* in the last isolates (DL). The analysis took into consideration either the antibiotics received by the patient during treatment (after collection of the first isolate; DL, black bars) or all antibiotics received by the patient (before collection of the initial isolate and/or during treatment; D0/DL, grey bars). CIP, ciprofloxacin; MEM, meropenem; TZP, piperacillin/tazobactam; FEP, cefepime; AMK, amikacin. Values >1 denote a potential association between previous use of the corresponding antibiotic and overexpression of the considered gene. The corresponding *P*-values are indicated on the right.

## 4. Discussion

To the best of our knowledge, this study represents one of the first efforts to examine in a systematic way the relationship between exposure to antibiotics in acute infection and the selection of resistance mediated by the four major multidrug efflux systems in *P. aeruginosa*. Our data show that overexpression of efflux was not only detected in a large proportion of isolates but that it contributes to an increase in median MICs to values above the clinical susceptibility breakpoints. This conclusion stems from two convergent observations.

First, there was an increase in the proportion of isolates overexpressing *mexA* and *mexX*, which is consistent with the fact that most patients have been treated with one or several of the antibiotics that are substrates of the corresponding transporters. In the initial isolates (D0 samples), *mexX* overexpression was the most prevalent, as reported by others [11,12]. However, *mexA* and *mexX* overexpression were equally distributed in the last isolates (DL) from our patient population, and even simultaneously overexpressed in the same isolates. This could result from selection of concomitant mutations in the regulators of these genes [13]. Some antibiotics, however, appear more prone to trigger a wide overexpression of genes coding for efflux transporters than others. Thus, exposure to the universal substrate ciprofloxacin resulted in the overexpression of *mexA* and *mexX*, whilst meropenem and amikacin (preferential substrates of MexAB–OprM and MexXY–OprM, respectively) selected predominantly for the overexpression of *mexA* for meropenem and *mexX* for amikacin. In this study, exposure of the patients to TZP did not appear to cause *mexA* overexpression even though piperacillin is preferentially transported by MexAB–OprM [14]. Future research may need to examine whether piperacillin may select for overexpression of *mexA* and/or whether tazobactam could interfere with this process. The situation may be more complex for cefepime, which causes a simultaneous overexpression of both *mexA* and *mexY*, perhaps consistent with the fact that, like other β-lactams, it is transported by MexAB–OprM while being also described as a substrate of MexXY–OprM [15]. Of note, the proportion of isolates showing expression of *mexC* or *mexE* was low in the D0 isolates and did not increase as unique genes in DL isolates. This is consistent with the fact that ciprofloxacin is described as the main selective agent associated with the expression of these genes [16,17] and only a few patients received this antibiotic. Conversely, a few DL isolates showed overexpression of *mexA* and/or *mexX* together with expression of *mexC* and/or *mexE* (or even all four

genes together), which, to our knowledge, has not yet been reported in clinical strains; a recent study with non-cystic fibrosis isolates from Thailand showed simultaneous overexpression of up to four different Mex systems including *mexA*, *mexX* and *mexE*, but not *mexC* [18].

As far as we could ascertain, an increase in antibiotic efflux upon exposure to substrates in *P. aeruginosa* has been mainly described in vitro. However, one case report documents the acquisition of a fluoroquinolone resistance phenotype during treatment, which was ascribed to a combination of target mutation and overexpression of MexAB–OprM [19]. Likewise, treatment with β-lactams has been reported to cause OprM overexpression, but neither the inducer nor the transporter involved were identified [20]. In the current study, confounding factors, such as the frequent occurrence of combined drug regimens, may explain the lack of association between piperacillin or cefepime exposure and overexpression of efflux. Moreover, substrates shown to cause increased efflux in vitro may not necessarily express the same properties in vivo. Whilst this could be tested in well-designed animal experiments, it is beyond the scope of the present study.

The second critical observation is that overexpression of genes encoding for antibiotic transporters is associated with an increase in the proportion of isolates categorised as clinically resistant using EUCAST interpretive criteria in most of the cases. Although these remain in the non-resistant range when considering CLSI criteria, we may need to revisit the long-held concept that efflux does not play a significant role in clinical practice because it is generally believed to confer only low-level resistance (see [5] for review and references cited therein). Thus, recent surveys showed that the prevalence of *mexB* or *mexY* overexpression is highest among meropenem- and tobramycin-non-susceptible isolates, respectively [21], and that overexpression of multiple efflux systems can be associated with multidrug resistance in isolates from non-cystic fibrosis patients [18]. On the same line, examination of collections of clinical strains showed that overexpression of *mexA* is a major contributing factor in resistance to meropenem, whilst overexpression of *mexX* causes resistance to cefepime or aminoglycosides [15,22,23]. Yet, and in contrast to what has been previously observed for laboratory mutants overexpressing both the MexAB–OprM and MexXY–OprM efflux systems [13], we did not systematically detect higher level resistance in strains overexpressing simultaneously *mexA* and *mexX* compared with isolates in which only one of these two genes was overexpressed. Equally intriguingly, we noticed large increases in the MICs of TZP for strains overexpressing *mexX* and of cefepime for strains overexpressing *mexA*, illustrating that these antibiotics may be more avidly transported by MexAB–OprM and MexXY–OprM than anticipated. In this context, cefepime has already been described as a substrate of MexAB–OprM and MexCD–OprJ [24].

This work suffers from limitations related both to the experimental and the clinical set-ups of the work. First, we only looked for changes in the expression of genes encoding for key components of the transporters studied. These changes may not necessarily predict commensurate changes in protein amounts and in effective transport activity (we did not test for changes in MIC using efflux pumps inhibitors, but this has been done previously on a limited number of laboratory and clinical isolates and showed that these parameters were well correlated [25]). Whilst proteomic and functional studies would have been desirable, these could not be undertaken at this stage. Also, in the context of the current study we could not examine the role of adaptive resistance in the MIC increases observed for aminoglycosides. Second, we were limited in our approach by the actual management of patients with severe infections and hospitalised in ICUs, which most often involves the administration of several antibiotics as well as of many other drugs. Thus, patients not only often received antibiotics before collection of the first isolate but, for the majority of them, were treated with several antibiotics either in combination or successively. Third, we could also not exclude that other non-antibiotic drugs could have triggered the overexpression of efflux systems; such effects, however, are mainly described in vitro and at supratherapeutic concentrations [26,27]. Moreover, MIC values may have been influenced by other mechanisms than efflux, which were not studied here. These difficulties are the price to pay for working with isolates obtained from patients as they are treated today, so as to ensure the potential clinical significance of the observations. Lastly, the present study did not examine other mechanisms of resistance that may contribute to the overall non-susceptibility of the organisms. As explained in the Introduction, this work specifically focused on efflux and should be complemented with a study of other mechanisms, which we hope to be able to present in a future report.

Despite these limitations, the present study may nevertheless lead to meaningful conclusions for both the clinical microbiologist and the clinician. First, we documented that active efflux is a frequent mechanism involved in multidrug resistance in *P. aeruginosa* in patients with acute infection and limited treatment duration. Second, since efflux selected during treatment can confer cross-resistance between antibiotics of different pharmacological classes, there is a clear necessity to closely monitor the susceptibility patterns of the isolates during therapy and to use appropriate diagnostic methods. Third, since overexpression of efflux systems was occurring during standard-of-care therapy, using recommended and widely accepted antibiotic dosages and schedules of administration, we may need to critically revisit these recommendations, paying further attention to other parameters than serum concentrations, as proposed for setting susceptibility breakpoints [28]. This could include measurement of the actual levels of penetration of the antibiotics at the site of infection [29] and an assessment of how this favours or disfavours the emergence of efflux-mediated resistance. In a broader context, by decreasing the intrabacterial concentration of antibiotics, efflux may favour the effective expression of other mechanisms of resistance, as shown for enzyme-mediated resistance to aminoglycosides [30] and, thereby, participate in the demise of our antibacterial armamentarium.

## Acknowledgments

The authors thank Prof. Y. Glupczynski (Laboratoire de microbiologie, CHU Dinant-Mont-Godinne, Yvoir, Belgium) for numerous suggestions and critical reading of the manuscript. The authors also thank the research nurses for help in collecting clinical and antibiotic usage data, and the clinical microbiologists (D. Piérard at Universitair Ziekenhuis Brussel, Brussels, A. Dediste at Laboratoire de Microbiologie, CHU Saint-Pierre, Brussels, and A. Simon at Laboratoire de Microbiologie, Cliniques Universitaires St-Luc, Brussels, Belgium) and the infectious diseases specialist (F. Jacobs at Hôpital Erasme, Brussels, Belgium) for having facilitated access to the isolates. Virginie Mohymont, Charlotte Misson and Jonathan Gesels provided dedicated technical help.

*Funding*: MR was the successive recipient of post-doctoral fellowships from the Région de Bruxelles-Capitale/Brussels Hoofdstedelijk Gewest (within the context of the programme 'Research in Brussels') and the Belgian Fonds de la Recherche Scientifique (F.R.S.-FNRS). FVB is Maître de recherches of the Belgian Fonds de la Recherche Scientifique (F.R.S.-FNRS). This work was supported by the Région de Bruxelles-Capitale/Brussels Hoofdstedelijk Gewest and the Belgian Fonds de la Recherche Scientifique Médicale (F.R.S.M.) [Grant Nos. 3.4597.06, 3.4583.08 and 3.4639.09; general funding and partial support of SC].

*Competing interests*: None declared.

*Ethical approval*: The study protocol was approved by the Ethical Committee of the Faculty of Medicine of the Université catholique de Louvain (Brussels, Belgium) within the context of a grant application to the Belgian Fonds de la Recherche Scientifique Médicale (F.R.S.M.) [Grant No. 3.4597.06].

## References


[1] Sykes R. The 2009 Garrod lecture: the evolution of antimicrobial resistance: a Darwinian perspective. J Antimicrob Chemother 2010;65:1842–52.
[2] Quinn JP, Dudek EJ, DiVincenzo CA, Lucks DA, Lerner SA. Emergence of resistance to imipenem during therapy for *Pseudomonas aeruginosa* infections. J Infect Dis 1986;154:289–94.
[3] Riou M, Carbonnelle S, Avrain L, Mesaros N, Pirnay JP, Bilocq F, et al. In vivo development of antimicrobial resistance in *Pseudomonas aeruginosa* strains isolated from the lower respiratory tract of intensive care unit patients with nosocomial pneumonia and receiving antipseudomonal therapy. Int J Antimicrob Agents 2010;36:513–22.
[4] Morita Y, Sobel ML, Poole K. Antibiotic inducibility of the MexXY multidrug efflux system of *Pseudomonas aeruginosa*: involvement of the antibiotic-inducible PA5471 gene product. J Bacteriol 2006;188:1847–55.
[5] Van Bambeke F, Glupczynski Y, Plesiat P, Pechere JC, Tulkens PM. Antibiotic efflux pumps in prokaryotic cells: occurrence, impact on resistance and strategies for the future of antimicrobial therapy. J Antimicrob Chemother 2003;51:1055–65.
[6] Vettoretti L, Plesiat P, Muller C, El Garch F, Phan G, Attree I, et al. Efflux unbalance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. Antimicrob Agents Chemother 2009;53:1987–97.
[7] Stover CK, Pham XQ, Erwin AL, Mizoguchi SD, Warrener P, Hickey MJ, et al. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. Nature 2000;406:959–64.
[8] Clinical Laboratory Standard Institute, Document S100-S24 Performance standards for antimicrobial susceptibility testing; twenty-fourth informational supplement. Wayne, PA: CLSI; 2014.
[9] Dumas JL, van Delden C, Perron K, Kohler T. Analysis of antibiotic resistance gene expression in *Pseudomonas aeruginosa* by quantitative real-time-PCR. FEMS Microbiol Lett 2006;254:217–25.
[10] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
[11] Fehlberg LC, Xavier DE, Peraro PP, Marra AR, Edmond MB, Gales AC. β-Lactam resistance mechanisms in *Pseudomonas aeruginosa* strains causing bloodstream infections: comparative results between Brazilian and American isolates. Microb Drug Resist 2012;18:402–7.
[12] Vatcheva-Dobrevska R, Mulet X, Ivanov I, Zamorano L, Dobreva E, Velinov T, et al. Molecular epidemiology and multidrug resistance mechanisms of *Pseudomonas aeruginosa* isolates from Bulgarian hospitals. Microb Drug Resist 2013;19:355–61.
[13] Llanes C, Hocquet D, Vogne C, Benali-Baitich D, Neuwirth C, Plesiat P. Clinical strains of *Pseudomonas aeruginosa* overproducing MexAB–OprM and MexXY efflux pumps simultaneously. Antimicrob Agents Chemother 2004;48:1797–802.
[14] Masuda N, Gotoh N, Ishii C, Sakagawa E, Ohya S, Nishino T. Interplay between chromosomal β-lactamase and the MexAB–OprM efflux system in intrinsic resistance to β-lactams in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 1999;43:400–2.
[15] Hocquet D, Nordmann P, El Garch F, Cabanne L, Plesiat P. Involvement of the MexXY–OprM efflux system in emergence of cefepime resistance in clinical strains of *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 2006;50:1347–51.
[16] Morero NR, Monti MR, Argarana CE. Effect of ciprofloxacin concentration on the frequency and nature of resistant mutants selected from *Pseudomonas aeruginosa mutS* and *mutT* hypermutators. Antimicrob Agents Chemother 2011;55:3668–76.
[17] Kohler T, Michea-Hamzehpour M, Plesiat P, Kahr AL, Pechere JC. Differential selection of multidrug efflux systems by quinolones in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 1997;41:2540–3.
[18] Poonsuk K, Tribuddharat C, Chuanchuen R. Simultaneous overexpression of multidrug efflux pumps in *Pseudomonas aeruginosa* non-cystic fibrosis clinical isolates. Can J Microbiol 2014;60:437–43.
[19] Le Thomas I, Couetdic G, Clermont O, Brahimi N, Plesiat P, Bingen E. In vivo selection of a target/efflux double mutant of *Pseudomonas aeruginosa* by ciprofloxacin therapy. J Antimicrob Chemother 2001;48:553–5.
[20] Ziha-Zarifi I, Llanes C, Kohler T, Pechere JC, Plesiat P. In vivo emergence of multidrug-resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system MexA–MexB–OprM. Antimicrob Agents Chemother 1999;43:287–91.
[21] Cabot G, Ocampo-Sosa AA, Tubau F, Macia MD, Rodriguez C, Moya B, et al. Overexpression of AmpC and efflux pumps in *Pseudomonas aeruginosa* isolates from bloodstream infections: prevalence and impact on resistance in a Spanish multicenter study. Antimicrob Agents Chemother 2011;55:1906–11.
[22] Campo Esquisabel AB, Rodriguez MC, Campo-Sosa AO, Rodriguez C, Martinez-Martinez L. Mechanisms of resistance in clinical isolates of *Pseudomonas aeruginosa* less susceptible to cefepime than to ceftazidime. Clin Microbiol Infect 2011;17:1817–22.
[23] Aghazadeh M, Hojabri Z, Mahdian R, Nahaei MR, Rahmati M, Hojabri T, et al. Role of efflux pumps: MexAB–OprM and MexXY(–OprA), AmpC cephalosporinase and OprD porin in non-metallo-β-lactamase producing *Pseudomonas aeruginosa* isolated from cystic fibrosis and burn patients. Infect Genet Evol 2014;24:187–92.
[24] Jeannot K, Elsen S, Kohler T, Attree I, van Delden C, Plesiat P. Resistance and virulence of *Pseudomonas aeruginosa* clinical strains overproducing the MexCD–OprJ efflux pump. Antimicrob Agents Chemother 2008;52:2455–62.
[25] Mesaros N, Glupczynski Y, Avrain L, Caceres NE, Tulkens PM, Van Bambeke F. A combined phenotypic and genotypic method for the detection of Mex efflux pumps in *Pseudomonas aeruginosa*. J Antimicrob Chemother 2007;59:378–86.
[26] Tavio MM, Vila J, Perilli M, Casanas LT, Macia L, Amicosante G, et al. Enhanced active efflux, repression of porin synthesis and development of Mar phenotype by diazepam in two enterobacteria strains. J Med Microbiol 2004;53:1119–22.
[27] Tavío MM, Aquili VD, Fàbrega A, Vila J, Poveda JB. Overexpression of the quorum-sensing regulator *sdiA* and *soxS* is involved in low-level multidrug resistance induced in *Escherichia coli* AG100 by haloperidol, diazepam and NaCl. Int J Antimicrob Agents 2012;39:91–3.
[28] Mouton JW, Brown DFJ, Apfalter P, Cantón R, Giske CG, Ivanova M, et al. The role of pharmacokinetics/pharmacodynamics in setting clinical MIC breakpoints: the EUCAST approach. Clin Microbiol Infect 2012;18:E37–45.
[29] Boselli E, Breilh D, Cannesson M, Xuereb F, Rimmele T, Chassard D, et al. Steady-state plasma and intrapulmonary concentrations of piperacillin/tazobactam 4 g/0.5 g administered to critically ill patients with severe nosocomial pneumonia. Intensive Care Med 2004;30:976–9.
[30] Morita Y, Tomida J, Kawamura Y. Primary mechanisms mediating aminoglycoside resistance in the multidrug-resistant *Pseudomonas aeruginosa* clinical isolate PA7. Microbiology 2012;158:1071–83.